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Antithrombotic Benefits and Hemorrhagic Risks of Direct Thrombin Antagonists

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Heart attacks and strokes respond incompletely to aspirin and heparin (1–3). Other aspirin- and heparin- resistant arterial thrombo-occlusive processes occur as thrombotic complications of interventional procedures used in the treatment of symptomatic atherosclerotic disease, including thrombolytic reperfusion for acute coronary thrombosis, angioplasty, various types of atherectomy, placement of endovascular stents, endarterectomy, and implantation of small caliber vascular grafts (4–6). Under these conditions resistance to aspirin and heparin is attributable to the primary role of bound thrombin in mediating platelet recruitment (Fig. 1), a process not inhibited by aspirin's blockade of endoperoxide/thromboxane A_2 generation (7,8), and a conformation of thrombin not readily inactivated by heparin:antiplasmin complexes (9–12). Heparin is also neutralized locally by factors secreted from activated platelets, particularly platelet factor 4 (PF4) (13). Thus, thrombo-occlusive processes requiring more efficacious intervention than aspirin or heparin are thrombin-mediated, platelet-dependent and initiated by denudation/disruption of diseased arteries (Fig. 1) (14).

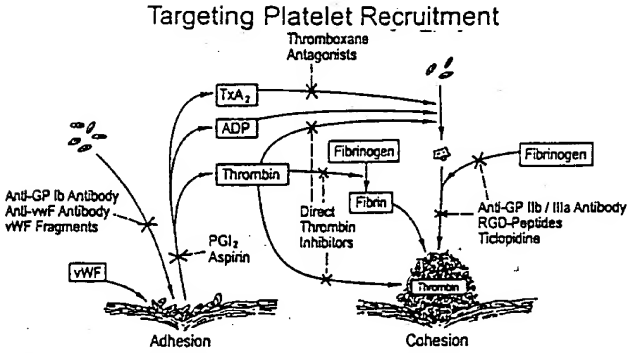

Fig. 1. Central role of thrombin in platelet recruitment. Both soluble and bound thrombin activate platelets at concentrations less than that required to convert fibrinogen to fibrin. Platelet cohesion is mediated through adhesive protein bridging between GPIIb/IIIa-receptors on adjacent activated platelets. Thrombin production on phosephatidylserine-rich activated platelet surfaces is amplified 300,000-fold via the tissue factor pathway. Fibrin consolidates the enlarging thrombolytic mass and binds with catalytically active thrombin. Aspirin inhibits the thromboxane A2 (TxA2) pathway of platelet recruitment by irreversibly inactivating cyclooxygenase. Triclopidine blocks all pathways of platelet recruitment, perhaps by modifying membrane signal transduction. Antithrombins, such as hirudin, inhibit thrombin-induced platelet activation and fibrin generation. Inhibitors of GPIIb/IIIa-dependent platelet receptors prevent the recruitment of platelets into thrombus.

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Accordingly, strategies for treating already established thrombus depend upon directly interrupting platelet recruitment by: a) inhibiting thrombin, the principal agonist (11,15,16); or b) blocking platelet glycoprotein (GP)IIb/IIIa receptor-dependent cohesion (17,18). For the prevention of resistant arterial thrombosis additional approaches may be used, including: c) inhibition of thrombin production by interfering with earlier reactions in the coagulation sequence (14); and d) impairment of vascular wall thrombogenicity (19) (Table 1).

In developing more effective antithrombotics it is critical to assess the corresponding hemorrhagic risks. Hemostatic safety is particularly important when therapy is associated with invasive procedures, thrombolytic treatment, or some underlying bleeding disorder. Recent clinical trials using antibody directed against platelet glycoprotein GPIIb/IIIa for high risk angioplasty (20), and hirudin for coronary artery syndromes (21,22) illustrate how troublesome hemorrhagic adverse effects may be when using these novel therapies. In the present discussion the relative efficacy and safety of inhibiting thrombin vs GPIIb/IIIa-dependent platelet cohesion are compared experimentally using a non-human primate model system. In addition, strategies are described for reducing hemorrhagic risks while safeguarding antithrombotic benefits.

Table 1. Antithrombotic strategies for resistant thrombosis.

Interruption of ongoing arterial thrombotic processes
1. Direct inactivation of soluble and bound thrombin
Natural antithrombin polypeptides
Synthetic antithrombin peptides
Irreversible inactivators of thrombin
Oral antithrombins
2. Platelet GPIIb/IIIa-receptor antagonists
Natural anti-platelet receptors polypeptides
Platelet GPIIb/IIIa receptor monoclonal antibodies
Synthetic peptide platelet GPIIb/IIIa receptor antagonists
Oral platelet GPIIb/IIIa-receptor antagonists
Prevention of Resistant Arterial Thrombotic Processes
3. Inhibition of thrombin production
Activated Protein C
Protein and peptide inhibitors of fXa
Inhibitors of tissue factor pathway
4. Impairment of vascular wall thrombogenicity
Dietary n-3 fatty acids
Acute restoration of endothelium

Targeting platelet recruitment

Following denuding arterial injury platelets and plasma coagulation factors rapidly interact with exposed vascular tissue factor and connective tissue structures under high-flow conditions leading to the formation of localized mechanical thrombotic masses in flow-dependent patterns variably composed of deposited platelets, insoluble fibrin, leukocytes, and entrapped erythrocytes (Fig. 1). Platelets initially attach to subendothelial connective tissue structures via von Willebrand factor tethered to platelet glycoprotein (GP)Ib/IX receptors (23). Platelets subsequently spread, express functional GPIIb/IIIa receptors for adhesive molecules (primarily fibrinogen), resulting in cohesion with ambient platelets (17,18,24). Tissue factor exposed at sites of vascular damage triggers the activation of coagulation zymogens, leading to the rapid generation of thrombin (25–27), which proteolytically activates platelets (11,28) and cleaves fibrinogen. Thrombin is the principal mediator of platelet activation at sites of arterial injury, leading to self-amplifying platelet recruitment which involves two supplementary agonists, adenosine diphosphate (ADP, secreted from storage granules of activated platelets), and thromboxane A_2 (arises via platelet arachidonic acid metabolic pathways) (7,9). Activated platelets further amplify thrombin production by expressing phosphotidylserine-rich membrane surfaces that promote the assembly of coagulation enzyme-cofactor complexes (29). Thrombin binds with fibrin in the forming thrombus; bound thrombin is susceptible to inactivation by direct inhibitors of thrombin, but resists the inhibitory effects of heparin or its derivatives (10,12,30). The formation of thrombus is localized to sites of vascular injury by several mechanisms, including: a) platelet attachment restricted to sites of endothelial denudation; b) tissue factor, a transmembrane protein, expressed only at sites of vascular disruption; and c) thrombus extension or propagation prevented by plasma- and vessel wall-dependent inhibitory pathways, including the inactivation of thrombin (by complexing with plasma antithrombin through endothelial heparin-like glycosaminoglycans), the activation of protein C via endothelial thrombomodulin and thrombin-induced production of prostacyclin, nitric oxide, and tissue plasminogen activator (7).

Direct antithrombins

Since bound thrombin mediates the recruitment of platelets into thrombus forming at sites of arterial injury, it constitutes the relevant target for developing more effective antithrombotic agents (Fig. 1). Thrombin's structural domains, including the catalytic site and two flanking clusters of accessory binding domains, regulate thrombin's interactions with inhibitors (14,16,31). Cleavage–dependent activation of the platelet thrombin receptors and proteolytic conversion of fibrinogen to fibrin depend on binding closely related non-catalytic exosite domains (32–34). Inhibition by plasma antithrombin:heparin complex requires binding with an accessory domain on the opposing facet of the thrombin molecule that is also closely related to the domain binding fibrin, thereby explaining the resistance of fibrin- bound thrombin to inactivation by heparin.

There is growing interest in developing direct antithrombins as potential antithrombotic agents (Tables 1 and 2) (14). Direct antithrombins include naturally occurring antithrombin peptides (35), synthetic competitive and irreversible antithrombin peptides (9,36–38), and chemical analogs that exhibit systemic activity after oral administration (39). Direct antithrombins inactivate both bound and soluble thrombin, and are therefore capable of interrupting platelet recruitment in aspirin- and heparin-resistant thrombotic processes developing at sites of arterial damage, albeit at doses substantially greater than that required to inactivate soluble thrombin (9–11,15). In experimental animals direct antithrombins are more effective than heparin in models of venous and arterial thrombosis and prevent rethrombosis following tissue plasminogen activator–induced thrombolysis (11,15, 36,40–43).

Recombinant hirudin, cloned from the medicinal leech, *Hirudo medicinalis*, binds reversibly but tightly with thrombin, forming an inhibitory stoichiometric complex with a dissociation constant of 20 fmol *in vitro* and *in vivo* (44). In experimental animals relatively low doses of hirudin inhibit the formation of venous thrombi and block intravascular coagulation when administered intravenously or subcutaneously (45). At somewhat higher doses hirudin is much more effective than high dose heparin and aspirin in reducing platelet deposition and thrombosis after angioplasty in pigs (46). Hirudin also interrupts platelet–dependent thrombus formation at sites of mechanical arterial injury in pigs and nonhuman primates, although the doses required to inhibit platelet deposition also produce corresponding impairment in hemostatic function (Table 2) (15,40). Hirudin is currently in randomized phase II/III clinical trials assessing its effect in coronary artery syndromes. Recently, the dosage levels have been decreased in these trials because of unexpectedly high rates of serious bleeding events (21,22). Thus, because hirudin produces dose-limiting antihemostatic effects, is immunogenic, may accumulate over time when administered subcutaneously, and has no known antidote, other antithrombin strategies may ultimately prove to be clinically more useful.

Antithrombin peptides based on arginine, benzamidine and hirudin exhibit antithrombin activities that are variable in potency and antithrombotic potential (Table 2). The tripeptide D-PHE-PRO-ARG inhibits thrombin-induced platelet aggregation and cleavage of fibrinogen *in vitro* and *in vivo*, demonstrating that inhibition of the catalytic site is alone sufficient for blocking thrombin's effects *in vivo* (36,47). Conversely, the dodecapeptide derived from residues 53–64 of hirudin ("Hirugen") inhibits fibrinogen binding and subsequent fibrin generation *in vitro* and shows antithrombotic efficacy in models of venous thrombosis, but this exosite-targeted peptide fails to interrupt thrombin-mediated platelet-dependent thrombus formation in baboon models of high-flow arterial thrombosis (48).

The antithrombotic effects of the direct antithrombins were compared for thrombogenic segments of Dacron vascular graft incorporated into exteriorized chronic femoral A-V silastic shunts under arterial flow conditions in baboons. Thrombus formation was measured in real time by gamma camera imaging as rates of platelet deposition and fibrin accumulation in animals previously labeled with circulating ^{111}In–platelets and ^{125}I–fibrinogen. Dose response effects were measured for each antithrombin administered by continuous intravenous infusion. The results are expressed as the dose (ID_{50}) and corresponding plasma

Table 2. Antithrombotic and antihemostatic effects of direct antithrombins.

Agents	Antithrombotic effects		Antihemostatic effects at ID_{50} doses	
	ID_{50} (nmol/kg/min)	IC_{50} (nmol/mL)	BT (min)	APTT (sec)
Hirudin	5	0.8	12	130
D–FPRCH$_2$Cl	25	1.2	12	95
D–FP–boroArg	25	1.1	12	180
BAP	125	20	21	>300
D–FPRH	250	19	14	145
benzamidine type	350	27	10	250
argipidine	>800	>23	>30	>300

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concentrations (IC_{50}) decreasing thrombus formation by half. Bleeding time measurements were performed by the standard template technique and APTTs were determined using standard methods.

Bifunctional antithrombin peptides (BAPs) have been developed and evaluated in preclinical models (36,37,49). For example, the structural specificity of the hirudin dodecacarboxy sequence has been combined with the active–center specificity of the D-PHE-PRO-ARG-PRO tetrapeptide through a -(GLY)$_4$ bridge, to form the bifunctional antithrombin peptide known as "Hirulog™", which exhibits cooperative antithrombotic effects *in vivo* (36,50). As with hirudin, platelet and coagulant hemostatic functions are correspondingly impaired by BAP at antithrombotic doses (Table 2). An alternative BAP combines hirudin specificity with active–center specificity of the tripeptide using α-keto amide transition state mimetic resistant to degradation by thrombin, yielding greater potency and stability (37).

D-PHE-PRO-boroARG (D-FPRBOH) blocks thrombin's catalytic site through a transition-state mechanism, and exhibits antithrombotic effects in several different animal models of arterial thrombosis (51,52). Two additional synthetic antithrombin peptides, D-PHE-PRO-ARG-H and D-MePHE-PRO-ARG-H have anticoagulant and antiplatelet effects when administered intravenously and orally in a number of animal species (47). Argipidine (argatroban or MD-805), is relatively potent *in vitro* (53), but fails to inhibit platelet–dependent thrombus formation *in vivo* (Table 2). Benzamidine based compounds also exhibit significant antithrombotic effects *in vivo* (Table 2) (45). Antithrombins peptides are less specific than natural or bivalent antithrombin peptides. Specificity may be critical for achieving therapeutic efficacy without dose limiting toxicities.

The potencies of the direct thrombin inhibitors have been compared in a number of different animal models, and in relation to heparin, aspirin or platelet GPIIb/IIIa receptor antagonists in accelerating tPA induced thrombolysis or preventing reocclusion (41). In all of these studies the direct thrombin inhibitors proved to be more effective than the other antithrombotic agents. In a coronary thrombosis model in dogs, hirudin was more effective than heparin, aspirin or RGD-containing peptides in accelerating tPA-induced thrombolysis in which a six-fold prolongation of the APTT by heparin failed to prevent reocclusion (54). However, argipidine (argatroban) in doses that prolonged the APTT two to four fold was not effective in preventing coronary reocclusion in dogs. Using a rat aortic thrombosis model the effects of heparin, hirudin and the synthetic bivalent antithrombin peptide ("Hirulog") were compared regarding their ability to accelerate thrombolysis and prevent reocclusion following tPA-induced thrombolysis (41). Compared to saline control, heparin had no significant effect on time to reperfusion or reocclusion, while the direct antithrombins decreased the number of reocclusions and accelerated thrombolysis. The superiority of hirudin over heparin in preventing thrombosis during and after thrombolysis and in permanently inactivating clot-bound thrombin has also been demonstrated in a study using a rabbit jugular vein model (42).

To address the benefit-risk ratio of a direct antithrombin during invasive cardiologic procedure, a recent study has compared anticoagulation with heparin or the BAP, "Hirulog", during routine cardiac catheterization (55). Anticoagulant support for coronary angiography was achieved without increased risk of bleeding at the site of arterial puncture. An open-label, dose-escalation study of BAP ("Hirulog") for prevention of venous thrombosis following orthopedic surgery has demonstrated dose-dependent antithrombotic effects of the direct antithrombin (56). Based on historical controls in this well-studied patient population, no increased risk for hemorrhage has yet been observed at fully-active dosages. These encouraging results require confirmation

in large, randomized, controlled clinical studies. The synthetic antithrombin D-PHE-PRO-ARG chloromethyl ketone (D-FPRCH$_2$Cl or PPACK) is unique among the antithrombin III-independent, direct antithrombins because it irreversibly inactivates thrombin, both soluble and thrombus-bound (9,11). Recent crystallographic studies confirm the tight interactions of this molecule with thrombin's catalytic pocket in addition to its covalent derivatization of HIS-57 in the catalytic triad (57). Systemic infusions of D-FPRCH$_2$Cl into nonhuman primates interrupt platelet-rich, aspirin- and heparin-resistant thrombi on Dacron vascular grafts, vascular stents, and hemodialyzers (9,11,30,58). Transient 1 hr intravenous infusions of D- FPRCH$_2$Cl produce lasting interruption of platelet deposition at sites of surgical carotid endarterectomy by irreversibly inactivating thrombin generated by, and bound to, forming thrombus (9). Moreover, delaying D-FPRCH$_2$Cl infusions until surgical hemostasis becomes established obviates the bleeding complications caused by earlier initiation of therapy without compromising the antithrombotic benefits. The lasting antithrombotic benefits following brief systemic treatment with D-FPRCH$_2$Cl appear to result from the permanent inactivation of thrombin bound to, and saturating, any already formed thrombus, and possibly other bound serine proteases participating in blood coagulation. As predicted, D-FPRCH$_2$Cl, but not competitive antithrombins such as hirudin, interrupt subsequent thrombus formation after topical application at a site of established thrombus. The dose-limiting and long-term toxicities associated with systemically or locally administered D-FPRCH$_2$Cl have yet to be adequately investigated with respect to its potential as a systemic therapeutic agent in man.

Overall, the relative antithrombotic and antihemostatic effects of direct antithrombins have been compared, including recombinant hirudin, the irreversible antithrombin peptide D-PHE-PRO-ARG chloromethyl ketone (D-FPRCH$_2$Cl), the competitive antithrombin peptide D-PHE-PRO-boroARG (D-FPRBOH), the bifunctional antithrombin peptide (BAP) combining the catalytic site inhibitor sequence D-FPR and the carboxyterminal dodecapeptide of hirudin, benzamidine-based and arginine-based (argipidine) synthetic direct antithrombins. All direct antithrombins tested interrupt platelet and fibrin deposition in a dose-dependent manner that is profound at the highest doses for blood exposed to all thrombogenic surfaces tested (Table 2). Thrombotic occlusion is also prevented by all agents at effective doses. Moreover, all of the direct antithrombins tested inhibit platelet hemostatic function in concert with their antithrombotic effects (bleeding times show intermediate prolongation by doses that reduce thrombus formation by half) (Table 2). Moreover, the prolonged bleeding times correlate with the amount of surgical bleeding during experimental endarterectomy (Table 3). Thus, experimental studies investigating the direct antithrombins provide unequivocal evidence that platelet-dependent thrombotic and hemostatic processes are thrombin-mediated in the model systems used, and that direct antithrombins produce dose-dependent inhibition of arterial thrombus formation that greatly exceeds the minimal antithrombotic effects produced by heparin and aspirin. However, these direct antithrombins cause corresponding decreases in both platelet and coagulation hemostatic functions, signifying an increased risk for abnormal bleeding during therapy that requires careful evaluation in human studies.

The antithrombotic effects of different therapeutic strategies for inhibiting platelet recruitment were compared for surgical caratoid endarterectomy in baboons. Platelet deposition at sites of endarterectomy was measured in real time by ^{111}In-platelet imaging. Bleeding into the surgical endarterectomy site was measured by estimating total Hgb collected from surgical sites. Agents were administered by continuous systemic infusion at fully antithrombotic doses. APC= activated protein C. TAP= tick anticoagulant peptide. FVIIai= activated factor VII rendered noncatalytic by reacting with GLU-GLY-ARG chloromethyl ketone.

Oral antithrombins with gradually improving bioavaliability are currently being developed (38,39). Oral antithrombins would be useful in the outpatient management of acute deep venous thrombosis, substituting costly inpatient monitored heparin infusions, and as a potential alternative for warfarin in the chronic anticoagulant prevention of deep venous thrombosis and pulmonary embolism.

Platelet receptor antagonists

Platelet recruitment is dependent on the expression by activated platelets of functional receptors for fibrinogen, or other adhesive ligands, leading to calcium-dependent inter-platelet linkages (17,24). The expression of functional GPIIb/IIIa receptors following agonist stimulation permits binding with multiple adhesive ligand molecules (including fibrinogen, von Willebrand factor, fibronectin, vitronectin and thrombospondin), via ARG-GLY-ASP (RGD) integrin recognition sequence in the adhesive proteins interacting with this receptor. Platelet recruitment is inhibited by anti-GPIIb/IIIa monoclonal antibodies, by naturally occurring peptides containing RGD or dodecapeptide sequences, and by synthetic competitive analogs.

Inhibition of the platelet GPIIb/IIIa receptor by murine monoclonal antibodies (7E3–F [ab']$_2$ [7E3], 10E5–F[ab']$_2$, AP2 and LJ-CP8) has been shown in experimental models to prevent platelet thrombus formation after vascular injury and to significantly shorten the time to reperfusion using tPA after thrombotic coronary occlusion (18,59). In dogs with experimental coronary thrombosis, 7E3 F(ab')$_2$ accelerates initial thrombolysis and prevents rethrombosis (60). Achieving antithrombotic effects with murine monoclonal antibodies requires doses that essentially eliminate GPIIb/IIIa receptor-function on all circulating platelets, resulting in striking inhibition of platelet hemostatic function and substantial experimental bleeding at sites of tissue injury in nonhuman primates (Table 3) (61). Prolongation of the bleeding time in these studies correlates with surgical bleeding with experimental endarterectomy (Table 3). Thrombocytopenia also develops in nonhuman primates following the administration of some murine monoclonal antibodies (62). In patients, antithrombotic doses of these monoclonal antibodies also produce template bleeding times >30 min and occasionally serious thrombocytopenia, although severe spontaneous abnormal bleeding has not been reported in clinical trials to date. Studies using "humanized" anti-GPIIb/IIIa monoclonal antibodies in patients at risk of arterial thrombotic events are reportedly free of both bleeding events and significant thrombocytopenia, at doses that apparently exhibit antiplatelet effects in patients with unstable

Table 3. Effects of inhibiting thrombin production on platelet deposition and surgical bleeding.

Inhibitor	Bleeding time (min)	^{111}In–platelet deposition(platx10^9)	Surgical bleeding (mL)
None	4.0 ± 1.2	2.7 ± 0.22	1.5 ± 0.7
Anti–GPIIb/IIIa MoAb (10 mg/kg)	>30	0.3 ± 0.1	>100
D–FPRCH$_2$Cl (150 nmol/kg/min)	>30	0.2 ± 0.1	>100
Hirudin (8 mg/kg/hr)	19 ± 3	0.2 ± 0.1	79 ± 15
APC (5 mg/kg/hr)	8.1 ± 1.5	0.1 ± 0.01	23 ± 7.5
TAP (0.8 mg/kg/hr)	4.2 ± 0.7	0.2 ± 0.05	15 ± 7.0
FVIIai (1 mg/kg)	4.1 ± 0.5	0.2 ± 0.04	2 ± 0.05

The antithrombotic effects of different therapeutic strategies for inhibiting platelet recruitment were compared for surgical caratoid endarterectomy in baboons. Platelet deposition at sites of endarterectomy was measured in real time by ^{111}In–platelet imaging. Bleeding into the surgical endarterectomy site was measured by estimating total Hgb collected from surgical sites. Agents were administered by continuous systemic infusion at fully antithrombotic doses. APC= activated protein C. TAP= tick anticoagulant peptide. FVIIai= activated factor VII rendered noncatalytic by reacting with GLU–GLY–ARG chloromethyl ketone.

angina and myocardial infarction treated with thrombolytic agents. Humanized antiGPIIb/IIIa monoclonal antibodies reduce acute coronary complications following high-risk angioplasty (20,63). This positive trial provides important "proof-of-concept" regarding the clinical benefits of inhibiting platelet recruitment. As shown by this study, it is also important to concurrently assess hemorrhagic risks when evaluating more effective antithrombotic therapies, because abnormal bleeding is the expected principal adverse outcome. This concern is particularly relevant when antithrombotic therapy is combined with invasive procedures, thrombolytic treatment, or associated with systemic or local pathologic disorders predisposing to abnormal bleeding. Additionally, twelve hours of antibody administration was required to obtain lasting antithrombotic benefit, thereby prolonging the period of risk for bleeding in treated patients. Thus, this study demonstrates the clinical usefulness of administering anti-GPIIb/IIIa c7E3 monoclonal antibodies for aspirin- and heparin-resistant thrombotic complications of high risk angioplasty, although this benefit comes at the cost of significant abnormal bleeding. This positive outcome supports the rationale for developing small-molecule, orally active inhibitors of platelet GPIIb/IIIa-dependent recruitment.

A number of naturally occurring cysteine-rich single-chain polypeptides have been isolated from snake venoms that potently inhibit the binding of fibrinogen to GPIIb/IIIa receptors and abolish platelet aggregation. This group of RGD–containing peptides include trigramin, bitistatin, echistatin, kistrin, and applaggin (14). In experimental animals all of these polypeptides, referred to as disintegrins, produce potent dose-dependent inhibition of platelet aggregation ex vivo and thrombus formation *in vivo*. Accelerated tPA-induced thrombolysis with prevention of subsequent reocclusion has also been demonstrated experimentally with some of these polypeptides. For example, bitistatin augments the effect of heparin in accelerating thrombolysis and prevents reocclusion following tPA-induced thrombolysis in canine models of coronary thrombolysis.

In general, these biologic peptides effectively inhibit binding of all RGD-containing adhesive proteins with platelet GPIIb/IIIa receptors at affinities similar to monoclonal antibodies, although their effects are short-lived *in vivo*. The potency of these natural peptides is attributable to the structural conformation of the RGD sequence resulting from intramolecular disulfide crosslinking, as illustrated by trigramin. A peptide isolated from the southwestern pygmy rattle snake Sistrurus m. barbouri, known as barbourin, specifically inhibits the binding of adhesive proteins with human platelet GPIIb/IIIa, as opposed to integrins on other cells. This specificity is a consequence of substituting ARG by LYS, forming the unique recognition sequence KGD. The potential therapeutic usefulness of these natural occurring inhibitory polypetides appears to be compromised by their induction of transient thrombocytopenia and their immunogenicity.

GPIIb/IIIa-antagonist peptides have been synthesized and characterized *in vitro* and *in vivo* as competitive inhibitors of platelet GPIIb/IIIa binding with adhesive proteins (64–69). *In vitro* and *ex vivo* these peptides inhibit platelet aggregation in a dose-dependent manner, similar to the effects produced by monoclonal antibodies and RGD-containing naturally occurring polypeptides. The peptide mimetics produce short-acting antithrombotic effects in experimental models of thrombosis. Although linear peptide antagonists have generally lacked suitable potency, the recently synthesized cyclic peptides exhibit antiplatelet effects comparable to the naturally occurring polypeptides, presumably because the cyclic configuration of the synthetic peptides reproduces the three-dimensional structures of the binding domains present in the more complex disulfide-rich natural peptides. For example, the tetrapeptide analogue ARG-GLY-ASP-0-methyltyrosine amide prevents reocclusion caused by platelet-rich thrombi after successful tPA- induced

thrombolysis in the femoral arteries of dogs, and the cyclic heptapeptide MK-852 is an effective antithrombotic compound in experimental models of arterial thrombosis (68,69). Synthetic cyclic peptides containing the KGD sequence also potently inhibit the binding of human platelets with adhesive proteins but with greater specificity for platelet GPIIb/IIIa than the integrins on other cells (64). The effects of this RGD-dependent specificity on the peptide's relative antithrombotic efficacy and hemostatic safety are currently being studied. Clinical testing of nonpeptide platelet receptor antagonists is underway (70,71).

Ticlopidine is an effective oral antiplatelet agent that produces dose-dependent inhibition of GPIIb/IIIa-mediated platelet recruitment (72,73). It produces greater protection from vascular occlusive events than aspirin, (74) and is clinically useful (75–78) despite its troublesome side effects, which include neutropenia, diarrhea, hepatic dysfunction and skin rashes. Clopidogrel, an analog of ticlopidine producing fewer complicating adverse effects is currently under development (79). Orally active synthetic small-molecule inhibitors of GPIIb/IIIa-dependent platelet recruitment are actively being developed for chronic oral therapy. The steep dose-response of these agents for inhibiting GPIIb/IIIa-dependent activity and antithrombotic effects, and the close association between antihemostatic vs antithrombotic effects leads to questions regarding the hemostatic safety of such chronic therapy (Table 4).

Minimizing hemorrhagic risks

In treating aspirin- and heparin-resistant thrombus that has already formed, platelet recruitment may be interrupted by inhibiting the agonist, bound thrombin, using direct antithrombins, or by blocking GPIIb/IIIa-dependent platelet cohesion with platelet receptor antagonist (Table 1). However, both of these systemic therapies produce impairment in hemostatic function that is proportionate to their antithrombotic effects. There are a number of strategies for reducing the hemorrhagic risks of antithrombins, while sparing antithrombotic efficacy, as compared with platelet receptor antagonists (Table 3).

First, administer a minimal effective dose. Because the platelet receptor antagonists have steep dose responses, it is difficult to find doses that reduce antihemostatic effects without losing substantial antithrombotic benefits. By contrast, the antithrombins have substantially broader dose responses, permitting downward titration of the dose to preserve useful antithrombotic effects, while possibly lowering the hemorrhagic risks to acceptable toxicity levels. For platelet receptor antagonists complete antithrombotic effects correlate with complete interruption of platelet hemostatic function, as measured by the template bleeding time (Table 4).

Second, shorten the duration of therapy to the time required to attenuate significantly thrombogenicity at sites of vascular damage, thereby reducing the duration of hemostatic risk to a minimum. This principle is illustrated by studies comparing outcomes using hirudin vs platelet receptor peptide antagonist (Fig. 2a and 2b). In these studies, the systemic administration of hirudin for one hour interrupted thrombus formation during the period of infusion, but failed to prevent thrombus from forming after treatment was discontinued. However, three hours of hirudin administration produced lasting interruption of thrombus formation after termination of therapy. By contrast, three hours of antithrombotic doses of the platelet receptor peptide antagonist failed to prevent subsequent thrombus formation. Putative mechanisms for attenuating thrombogenicity include carpeting the sites of injury with spent, unresponsive platelets or leukocytes, enzyme degradation of subendothelial vascular constituents, or

Table 4. Effects of oral GPIIb/IIIa receptor antagonists.

	Baseline	3 hrs	6 hrs
Platelet count (^{3}/μL)	389 ± 86	351 ± 103	286 ± 71
Inhibition of platelet agg. (%)	0	98 ± 4	96 ± 9
LIBS expression (sites/plat)	1800 ± 600	8760 ± 1800	6400 ± 2700
Bleeding time (min)	3 ± 1	13 ± 9	27 ± 7
Platelet deposition (x10^9)	4.4 ± 1.3	2.0 ± 0.73	0.34 ± 0.22
bTG (IU/mL)	15.4 ± 2.2	10.1 ± 2.0	7.5 ± 1.9
Fibrin accumulation (mg)	1.98 ± 0.57	2.0 ± 0.73	1.60 ± 0.08
FPA (nM)	20.2 ± 6.1	22.8 ± 9.2	21.9 ± 14.7
TAT (ng/mL)	134 ± 16	146 ± 14	128 ± 15

The relationship is shown among inhibition of platelet aggregation, expression of ligand–induced binding sites (LIBS), bleeding time and platelet deposition onto segments of vascular graft interposed in femoral arteriovenous shunts in baboons (see legend to Table 2). Interruption of platelet deposition occurred only when platelet hemostatic function was abolished; i.e., bleeding time >30 min, not when aggregation was abolished. Note the generation of thrombin (↑ FPA, fibrin accumulation and ↑ TAT) was not decreased despite elimination of platelet deposition.

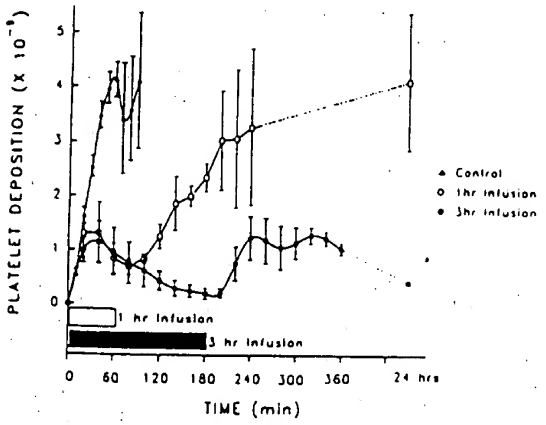

Fig. 2A. Antithrombotic effect of antithrombin and platelet receptor antagonist therapy. Whereas 1-hour hirudin in antithrombotic doses interrupts thrombus formation only during the hirudin infusion, 3-hr hirudin prevents subsequent thrombus formation.

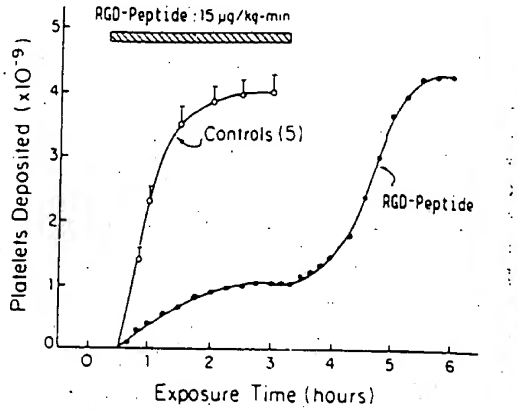

Fig. 2B. By contrast 3 hours of platelet receptor antagonist fails to produce lasting interruption of thrombus formation.

modulation of thrombogenic properties of accumulating leukocytes or proliferating vascular cells. For example, after three hours of continuous hirudin infusion the thrombogenicity at sites of vascular damage undergoes attenuation, as evidenced by a loss of bound thrombin and GPIIb/IIIa ligand-induced binding sites.

Third, administer antithrombotic treatment locally at thrombogenic sites in order to minimize systemic antihemostatic effects. One approach for achieving this goal is the use of a local delivery catheter with the design shown in Fig. 3.

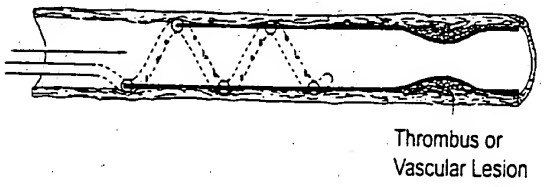

Fig. 3. Local delivery catheter.

This catheter delivers agents directly into the blood flow boundary layer, the slowest moving portion of flow profile immediately adjacent to vascular luminal surfaces. For antithrombin peptides, antithrombotic concentrations are achieved locally using less than one hundredth the dose required, to achieve that concentration by systemic administration. This is an important concept for achieving potent antithrombin levels at thrombogenic sites without producing detectable hemostatic effects systemically.

It is also important to avoid using potent antagonists of platelet recruitment in patients with known local or systemic hemostatic defects.

For the prevention of thrombus formation, agents may be chosen for development that selectively prevent vascular thrombus formation without compromising hemostatic plug formation. Antagonists of thrombin production, such as activated protein C, illustrate this latter strategy (Table 3). One of the potential mechanisms for reocclusion following successful thrombolysis is through exposure of tissue factor in the subendothelium and within lipid- and collagen-rich atherosclerotic plaques. In non-human primate models of thrombosis inactivated factor VIIa interrupts thrombus formation (Table 3) by inhibiting the tissue factor pathway (80). These encouraging findings suggest that the tissue factor pathway may be important in preventing thrombotic complications associated with elective interventional vascular procedures. Because of the importance of TF in thrombogenesis, peptides based on FVII binding sites or on tissue factor pathway inhibitor (TFPI) may interrupt thrombus formation without impairing hemostasis significantly.

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